Validity of Generalized Standard Addition Method for a Mixture of Amino Acid Analysis

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Abstract

A Modified version of the Generlized standard addition method (GSAM) was developed. This modified version was used for the quantitative determination of arginine (Arg) and glycine (Gly) in arginine acetyl salicylate – glycine complex . According to this method two linear equations were solved to obtain the amounts of (Arg) and (Gly). The first equation was obtained by spectrophotometic measurement of the total absorbance of (Arg) and (Gly) colored complex with ninhydrin . The second equation was obtained by measuring the total acid consumed by total amino groups of (Arg) and (Gly). The titration was carried out in non- aqueous media using perchloric acid in glacial acetic acid as a titrant. The developed method is accurate, precise, and free from interferences and may provide a useful approach to calibrate in the direct analysis of solid sample and it is suitable method to be used as a quality control procedure.

Key words: GSAM , amino acid analysis .

الخلاصة

تم ادخال تطوير على طريقة الأضافة القياسية العامة GSAM بشكل يُسهل أستخدامها لغرض التعيين الكمي للحوامض الأمينية الداخلة في تركيب المعقد أرجنين أسيتايل ساليسيليت – كلايسين وللحصول على معادلتين خطيتين تم دمج الطريقة اللونية (بأستخدام محلول النهدرين) للحصول على المعادلة الأولى وطريقة التسحيح في محيط لامائي (بأستخدام حامض البيركلوريك كمحلول مسحح) للحصول على المعادلة الثانية ،وذلك بعد عزل الأسيتايل ساليسيلك أسيد من المعقد بأضافة حامض الهيدروكلوريك(٠,٥عياري) وأستخلاص الأسيتايل ساليسيلك أسيد المتحرر بواسطة المذيب العضوي الأيثر وتقدير كميته بطريقة التسحيح (التسحيح المباشر أوالأرجاعي) . النتائج التي تم التوصل اليها دلت على كون هذه الطريقة سريعة والنتائج دقيقة وخالية من التداخلات الكيمياوية ولا تحتاج لأجهزة معقدة .

Introduction

Numerous methods for the analysis of acetylsalicylic acid could be found in the literature [1-10]. However, these methods are not suitable for the analysis of acetylsalicylic acid mixture with arginine and glycine. Argnine and glycine interfere with direct titrimetric determination of acetylsalicylic acid therefore, it was intended to separate acetylsalicylic acid quantitatively from its salts before it is determined by titrimetric method. It was intended to use spectrophotometry or titrimetry for determination of arginine and glycine . These two methods are simple and therefore the most widely used in routine drug and many compounds analysis as could be seen from the latest version of British , Swiss and United State Pharmacopeias. However, direct application of these methods for determination of arginine and glycine in a mixture containing both of them is not possible because they interfere with each other. As a remedy for this situation it was decided to use the GSAM which has wide applications [11-17]. It is based on the principle of varying both of the sample molar concentration and pH of solution. Aimed at the validation and standardization of analytical procedures with direct solid sample contain two types of amino acids without previous isolation. Many methods were used for the determination of arginine and other amino acid involved previous isolation like the classical analytical technique uses automated amino acid analyzers, However these methods require expensive dedicated equipment duo to the post- column derivatization of the amino acids , long assay times and large samples volumes [18] .Also the studies of PITC as the derivatizing agent, following the Pico-Tag method used for the determination of arginine & other amino acid , those studies require the establishment of standard–added curve (by application of the standard addition method)to avoid proportional errors [19]. The non-aqueous titration was used for detection of free amino group in amino acid [20].This method used for the quantitative determination of many chemical compounds and drugs in pharmaceutical forms, providing precise and accurate results, which could be verified by statistical methods [21-23]. A spectrophotometric method are widely used for determination of amino acid based on the reaction with coloring agent at different pH forming colored complex that measured at specific wavelength[24-27].

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Received : 11/5/2010
Accepted : 27/6/2010

Experimental
Material and Methods

Glacial acetic acid (99.8 %) was obtained from sigma. Standard perchloric acid (0.1 ml) in glacial acetic acid was prepared by diluting (4.25 ml) of perchloric acid (72%) to (500 ml) with glacial acetic acid containing (10 ml) acetic anhydride. The prepared solution was standardized by titration with standard anhydrous sodium carbonate (0.106 gm) in glacial acetic acid (50 ml) the titration end point was determined potentiometrically with potentiometric titration equipped with glass – calomel combined electrode. The prepared standard was used in the titrimetric procedure for the determination of arginine and glycine in arginine acetylsalicylate–glycine complex. Anhydrous sodium carbonate (99.5 %) , sodium hydroxide (0.5 M) and hydrochloric acid (0.5 M) were Aldrich standards . Arginine (98%, sigma) was standardized to determine the exact percentage of arginine by titration with standard hydrochloric acid (0.5 M). Glycine (99%, fluka) was used after drying in an oven for 1hr at (105ºC). Acetylsalicylic acid was purified by recrystilization from ethanol/ water mixture and standardized with standard (0.5 M) sodium hydroxide [10]. The determined percentage of acetylsalicylic acid was (99.4 %).The standards arginine , glycine and acetylsalicylic acid were used to prepare arginine acetylsalicylate – glycine complex . The later was used to test the validity of the proposed method. Sodium acetate buffer (4M, pH 5.5) was prepared by dissolving sodium acetate trihydrate NaOAc.3H2O (BDH "Analar "99.9) in 200 ml of deionized water. The solution was heated with stirring at (60ºC) until a clear solution was obtained. Glacial acetic acid (50 ml) was added and the volume was completed to (500 ml) with distilled water. The pH was adjusted by a drop wise addition of sodium hydroxide (4M) to pH (5.5). Ninhydrin reagent was prepared as following : ninhydrine (I gm) in 2 - methoxy ethanol (25 ml) and stannous chloride $SnCl_2$.2H2O (0.07 g) with continuous stirring until completely dissolved [28]. Sodium acetate buffer (8.3 ml) was added and the resulting solution was immediately transferred to dark glass reservoir bottle and a steam of nitrogen gas was babbled into the reagent solution for approximately (20 min). Titration was made with potentiometric titrator (Metrohm , Switzerland) titro process or equipped with(Metrohm).Dosimate and glass calomel combined electrodes. Spectrophotometric measurement was performed on carry (100 conc) uv- visible spectrophotometer using (1 cm) cells.

Determination of acetyl salicylic acid in this complex

(1gm) of arginine acetylsalicylate–glycine complex was weighed and dissolved in (25 ml) of distilled water. The salt was converted to free acetyl salicylic acid by acidification with hydrochloric acid (3 M) until pH (2.7) was reached. The acetyl Salicylic acid was extracted by ether (3 × 20 ml). The ether portions were pooled together and ether was evaporated by a rotary evaporator under vacuum. The acetyl salicylic acid was collected to be determined quantitatively by back titration method or by direct titration method [5, 10]. The result obtained for acetyl salicylic acid was corrected for the presence of salicylic acid as an impurity during liberating and separating acetyl salicylic acid form the complex. Salicylic acid was then determined in the liberated materials [29].

Determination of arginine and glycine in this complex

This was achieved by constructing and solving equations (2) and (4) using ninhydrin spectrophotometric and titrimetric method in non – aqueous media respectively.

Spectrophotometric Method

(36 mg) of the complex was precisely weighed and dissolved in (50 ml) distilled water to prepare a stock solution. An aliquot (1 ml) of this stock solution was diluted to (25 ml) with distilled water. An aliquot (1 ml) of this solution was mixed with (1ml) of ninhydrin reagent in a stoppered test tube , shacked and placed in a boiling water bath for (15 min). Ethanol (2.5 ml) and sodium acetate buffer (2.5 ml) were added to the mixture which was then cooled below (30 ºC). The sample was shacked thoroughly for (30 second) and absorbance was measured at (570 nm) against a reagent blank using (1 cm).Standard curves for arginine and glycine were constracted using (0.05 = 0.2 mM) standard solution for each. The values of the molar absorpitivity coefficient aA and aG in equation (2) were determined from slops of these calibration curves.

Titrimetric Method in non – aqueous media

(0.3 gm) of the complex was dissolved in glacial acetic acid (50 ml) in a dry flask. The glass electrode of the titroprocessor was immersed in the solution and the mixture was titrated against standard perchloric acid (reagent 1). A plot of pH against the titrant volume was constructed to obtain the titration end point.

Stability study

Identical aqueous solution (0.1 g/ 100 ml) of the complex was made. The stability was determined at different temperatures (25,

40, 50, 60 & 70 ºC) incubated in ovens for certain intervals. The decomposition of arginine acetylsalicylate – glycine complex was indicated by the release of acetylsalicylic acid , arginine and glycine as appears on TLC using a solvent system of (n-propanol : 34% ammonia 7 :3) . The appearance of three spots on TLC plates for acetyl salicylic acid, arginine and glycine when the samples were incubated at (50, 60 & 70) ºC . While incubation at (25 and 40) ºC showed only one spot for arginine acetylsalicylate – glycine complex.

Effect of Buffer

Glycine was used as a buffer to stabilize arginine acetylsalicylat complex. The concentration of glycine is (0.5 M) in respect to arginine (1M) to maintain the pH of the preparation at (4.7).

Result and Discussion

Method of calculation

The GSAM is a traditional vector – matrix notation to construct and to solve a system of (n) linear equation in order to determine the concentration of a mixture of (n) substances. The mixture of compounds interferes with each other when measured by (n) different sensors that belong to any suitable analytical technique. According to GSAM, the following system of two linear equations is required to determine arginine and glycine:

$$\left.\begin{aligned} A1 &= a11\%A + a12\ \%\ G \\ A2 &= a21\%A + a22\ \%\ G \end{aligned}\right\} \quad \ldots\ldots\ldots (1)$$

Where ; A1 and A2 are the total responses of two analytical sensors (1 and 2) to the percentage of arginine (% A) and glycine (% G) in the sample the factors a11 – a22 are absorptivity constant multiplied by the optical path length (i.e of the sample) .Theory of GSAM equations (1) gives precise result for %A and %G when the following two conditions are fulfilled: Firstly there should be a large difference in magnitude between the ratio a11/a12 and the ratio a21/a22. Secondly the precision is measuring A1 and A2 should be high. These requirements arise from the fact that the mathematical manipulation magnify the random error in measurement , so that large random error is produced in the calculated concentration of the measured substances (i.e %A and %G) [11,12] .According to the requirements stated in these two conditions, it was decided to construct one of the equations of the system by using spechtrophotometric technique, while the second equation is to be constructed using titrimetic method. Ninhydrin was chosen as a color developing reagent in the spechtrophtometric procedure, as it is the most selective among other coloring agent for spechtrophotometric determination for amino acids [30-31].The titrimetric method was used to obtain the second equation which could not be performed in aqueous medium because of the neutral behavior of salts in aqueous medium that prevent the occurrence of a clear titration end point . Accordingly, a titrimetric method in non- aqueous media was adopted using glacial acetic acid as a solvent and a solution of perchloric acid in glacial acetic acid as a titrant. This method was used for quantitative determination of salt of amines with carboxylic acids [32-33].The presence of an acidic salts in non-aqueous solvent behave as bases and therefore produce a clear titration end point when titrated with strong acid [34].This experiment of procedure was intended to produce two linear equations in which the ratio a11/a12 and a21/a22 are very different in magnitudes. This was expected from the fact that arginine and glycine have nearly the same efficiency to produce colored complexes with ninhydrin reagent in spectrophotometric procedure [31].While in the titrimetric procedure arginine molecules have two titratable amino groups in contrast to the glycine molecules which have one titratable group. The first equation which is obtained from the spectrophotometric procedure was derived starting from Lambert – beer law as follows :

$$\mathbf{A} = \frac{\mathbf{10aA\ b\ Wasg}}{\mathbf{MwA\ vo\ f1f2\ \ldots.fn}}\%\ \mathbf{A} + \frac{\mathbf{10aA\ b\ Wasg}}{\mathbf{MwG\ vo\ f1f2\ldots.fn}}\%\ \mathbf{G} \quad \ldots (2)$$

Equation (2) can be abbreviated as follows:

$$\mathbf{A = a11\ \%A + a12\ \%G} \quad \ldots\ldots\ldots (3)$$

In equation (2) Wasg gm of arginine acetyl salicylate – glycine complex sample was dissolved to prepare (vo ml) of stock solution. This stock solution was then subjected to serial dilutions (n) with f1,f2…fn dilution factors to reach the required final concentration.

$$\mathbf{Fn} = \frac{\mathbf{volume\ of\ the\ volumetric\ flask\ use}}{\mathbf{volume\ taken\ from\ the\ soluti}}$$

Mw A and Mw G are the molecular weight of arginine and glycine respectively. aA and aG are the molar absorpitivity constants of arginine and glycine respectively.The use of a

combination of titrimetry and spechtrophotometry satisfies the requirements stated in (2). The precision of the spectrophotometric method is moderate in general.While; the precision of the titrometric method is very high. Therefore, the use of non-aqueous titration method improves the overall precision of the results obtained from such combination of methods. The second equation, was obtained from titrimetric procedure as follows:

$$\mathbf{MpVp} = \frac{\mathbf{2Wasg \times 10}}{\mathbf{MwA}} \% \mathbf{A} + \frac{\mathbf{2Wasg \times 10}}{\mathbf{MwG}} \% \mathbf{G} \quad \ldots(4)$$

Equation (4) can be abbreviated as below:

MpVp = a21%A + a22%G …….. (5)

Equation (4) V ml of glacial acetic acid containing V asg of the analyzed arginine acetyl Salicylic-glycine complex was titrated with Mp molar standard of perchloric acid, so that Vp ml of the standard was required to reach the end point . Equations (3) and (5) are mathematically compatible and can be solved linearly. The ratio a11/a12 and a21/a22 have large differences in their magnitudes since a11/a12 ≈ 2 while a 21/a 22 ≈ 1.

Development of Titrimetric Procedure for Acetyl salicylic acid determination

The complex sample solution must be acidified to liberate acetyl salicylic acid form its salt with arginine before extraction by ether. The optimum pH of the acidified aqueous solution was determined experimentally by applying the sample complex at different pH values of the extracted sample solution. The result of this study indicates that pH (2.7) is the optimum pH for acetyl salicylic acid extraction. At pH higher than (2.7) low result for acetyl salicylic acid were obtained due to incomplete liberation acetyl salicylic acid from its salt with arginine.The extraction step is vital in the development procedure because an attempt to perform direct titration with hydrochloric acid without performing apparent titration end point as could be seen from (Figure 1).

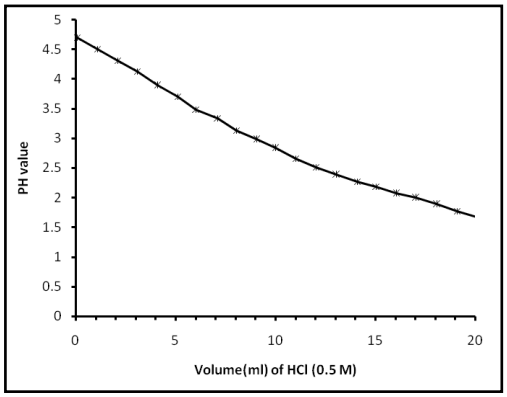

Figure 1: Titration curve of arginine acetylsalicylate -glycine solution with standard hydrochloric acid (0.5).

Analysis of Arginine Acetyl salicylate – Glycine complex

Quantitative Determination of Acetyl salicylic acid.

Acetyl salicylic acid was determined quantitatively by hydrolysis and back titration method [29]. The result of the two methods was summarized in table (1).

Table 1: result of acetylsalicylic acid, arginine and glycine in arginine acetyl salicylate – glycine complex

Item	Expected Percentage W/W	Calculated Percentage (w/w) ± SD	C.V
Acetylsalicylic Acid	50.0	49.6 ± 0.2 * 49.6 ± 0.3 *	0.4 % 0.6 %
Arginin	40.0	40.3 ± 0.6	1.5 %
Glycine	10.0	9.7 ± 0.2	2.0 %

* Hydrolysis and back titration method.

* *Direct titration method.

Quantitative determination of arginine and glycine in the complex

The quantitative determination of arginine and glycine in the complex using a modified version of the GSAM was achieved by a combination of colorimetric method (to obtain the first equation) and non-aqueous titration method (to obtain the second equation).The colorimetric method using the colored complex of arginine and glycine with ninhydrin was applied once at different pH media and once by using different wavelengths; the results of these experiments were summarized in table (2) and (3) and in figure (2) and (3) respectively.

Table 2: Molar absorptivity constant a* of arginine and glycine colored complex with ninhydrin at different pH values measured at 570nm

pH of ninhydrin solution	aA	aG	aA / aG
5.5	0.83	0.62	1.34
7.0	1.07	0.78	1.37
9.0	1.73	0.97	1.41

*Represent the Molar absorptivity multiplied by the cell width

Table 3: Molar absorptivity constant a* of arginine and glycine colored complex with ninhydrin at different wavelengths.

pH of ninhydrin solution	Wavelengths nm	aA	aG	aA/aG
5.5	244	1.19	0.92	1.29
	409	0.74	0.54	1.37
	570	0.83	0.63	1.32
7.0	244	1.28	0.97	1.32
	409	0.87	0.62	1.40
	570	1.02	0.75	1.36
9.0	244	1.40	1.08	1.30
	409	0.92	0.67	1.37
	570	1.32	1.00	1.32

*Represent the Molar absorptivity multiplied by the cell width

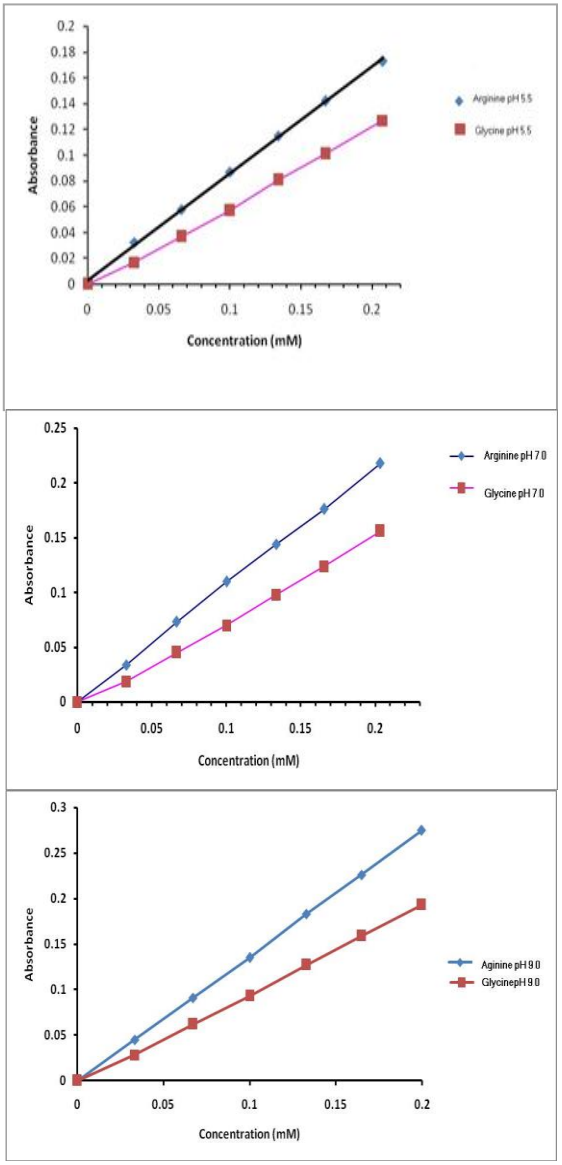

Figure 2: standard curve of arginine and glycine colored complexes with ninhydrin at different pH values measured at 570 nm

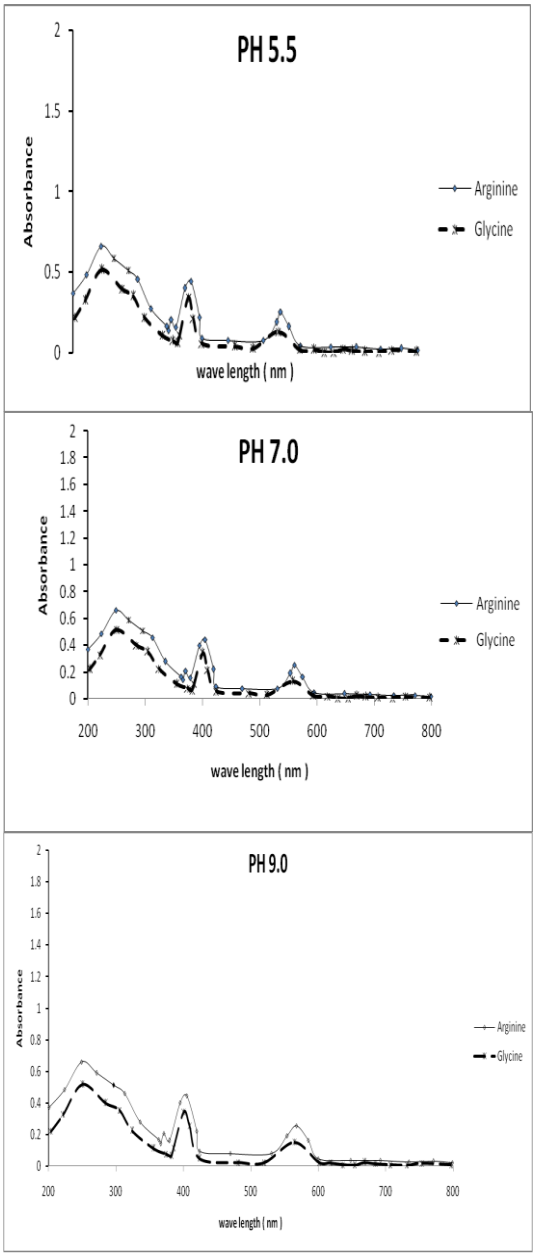

Figure 3: uv-visible spectra of arginine and glycine colored complexes with ninhydrin at different pH values

According to the non-aqueous titration method; the titration end point of the titration curve between standard perchloric acid with the complex was determined potentiometrically. A typical plot for the titration curve was exhibited in figure (4)

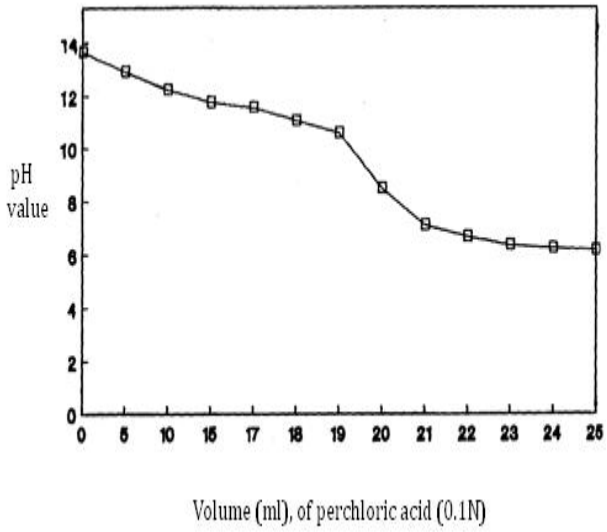

Figure 4: titration curve between standard perchloric acid (0.1N) and arginine acetylsalicylate –glycine complex.

The result of stability study indicates the appearance of three spots on TLC at (50, 60, 79)ºC and only one spot at (25, 40) ºC as summarized in table (4).

Table 4: *Rf values of the arginine acetylsalicylate – glycine complex at different temperatures for certain time intervals

C	Intial	1st	2nd	4th
25	0.42	0.42	0.42	0.41
40	0.42	0.42	0.42	0.41
50	0.42	0.43	0.46	0.55
60	0.42	0.44	0.45	0.57
70	0.42	0.45	0.55	0.56

*The solvent system is (n-propanol : 34% ammonia 7 :3)

Interference

As mentioned earlier the degradation product of arginine acetylsalicylate – glycine complex might interfere with the determination of arginine acetyl Salicylic acid and glycine by the proposed method. The evacuation step in the acetylsalicylic acid analysis procedure ensures complete removal of acetic acid thus prevents its influence on the titration of acetyl salicylic acid with sodium hydroxide. Beside, the determination of the amount of salicylic acid [20] and the subsequent correction eliminates its interference on the acetyl Salicylic acid determination. The non – aqueous titration procedure is not affected by all the degradation procedure as this procedure is selective for basic groups such as the amino group.

Conclusion

The proposed procedure is suitable to be used as a quality control procedure for the determination of arginine acetylsalicylate – glycine complex, as well as many formulas that contain essential and non essential amino acids (free or in combination)in different preparation like food supplement or other pharmaceutical preparation.This proposed procedure is simple, fast and accurate in compares with other procedures.

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